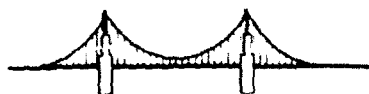


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INSTITUTE REPORT NO. 486

HEAT SHOCK PROTEIN INDUCTION IN HUMAN  
CELLS BY CO<sub>2</sub> LASER IRRADIATION

RONALD E. FERRANDO, STEVEN T. SCHUSCHEREBA, PHILLIP D. BOWMAN  
JULIE A. QUONG, JANET M. YANG, AND BRUCE E. STUCK

Division of Ocular Hazards

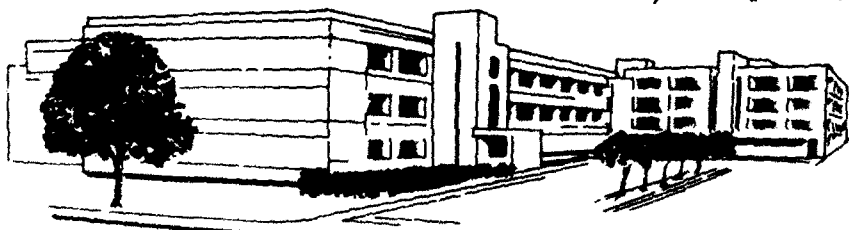
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Barbara A. Wilson  
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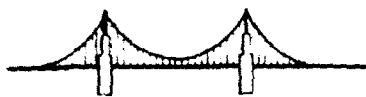


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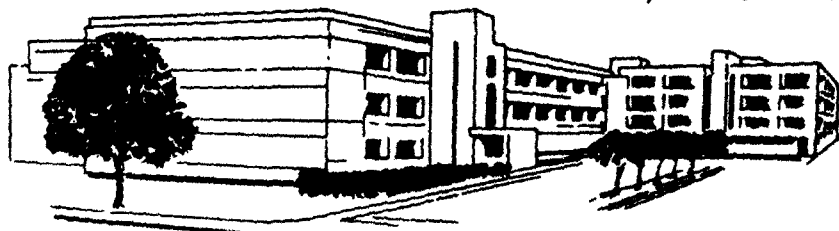
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HEAT SHOCK PROTEIN INDUCTION IN HUMAN CELLS BY CO<sub>2</sub>  
LASER IRRADIATION -- RONALD E. FERRANDO, STEVEN T.  
SCHUSCHEREBA, PHILLIP D. BOWMAN, JULIE A. QUONG, JANET  
M. YANG, AND BRUCE E. STUCK

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*for* Barbara A. Wilson, MAJ, MS, DCA  
John R. Hess (date) *14 June 93*  
COL, MC  
Commander

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## **ABSTRACT**

This study investigated the effect of millisecond exposures from a carbon dioxide (CO<sub>2</sub>) laser on the production of heat shock proteins in human fibroblast cell cultures. Heat shock or stress proteins (hsp) are induced to high levels by heat shock, transition series and heavy metals, amino acid analogs, certain chemicals, and ultraviolet radiation. These proteins are highly conserved throughout nature and their regulation and cellular functions are just beginning to be understood. The production of hsps was monitored by <sup>35</sup>S-methionine incorporation into newly synthesized heat shock protein and by immunolocalization. It was shown that CO<sub>2</sub> laser irradiation could induce the production of hsps. The time course of hsp synthesis and localization within cells was also determined. The hsp synthesis produced by the CO<sub>2</sub> laser was compared to the effects of sodium arsenite and heat. The results of this study indicate that hsp70 is the major heat shock protein induced by CO<sub>2</sub> laser radiation and that this response is most likely due to the thermal effects of the laser.

## **PREFACE**

The senior author of this study submitted this manuscript as a thesis to San Francisco State University in partial fulfillment for the degree of Master of Arts in Biology: Physiology and Behavioral Biology, awarded on August 23, 1992.

## **ACKNOWLEDGEMENTS**

I would like to thank Mr. Steven T. Schuschereba and Dr. Phillip D. Bowman for their continued assistance and encouragement throughout this project. I would also like to acknowledge Julie A. Quong, Janet M. Yang and Karen A. Semey for their technical support throughout this project.

Heat shock protein induction in human cells by CO<sub>2</sub> laser irradiation  
--Ferrando et al.

## INTRODUCTION

The heat shock response has generally been studied in the context of elevated temperature, oxidative stress, or heavy metals. Sublethal conditions induce this response in cells from many organisms. The proteins which are subsequently synthesized are referred to as heat shock proteins (hsps) or stress proteins.

The heat shock response was first described in 1962 as a response to a higher than normal temperature in polytene chromosomes in *Drosophila* salivary gland cells (1). Morphologically, this response was associated with a new chromosome puffing pattern, which indicated that a new class of genes was being expressed. Twelve years later Tissieres et al. demonstrated a correlation with de novo synthesis of a new class of proteins termed the hsps (2). Similar induction of hsp synthesis was later discovered in bacteria (3), chicken (4), and mammalian cells (5). Similar responses have been observed in all organisms studied thus far (6). It is now well established that in mammalian cells the family of proteins induced by stress consists of a series of proteins with approximate molecular weights of 28, 32, 56, 72/73, 90 and 110kD (7).

Heat shock proteins are highly conserved. For example, the human hsp72/73 is 73% homologous in its amino acid sequence to the *Drosophila* protein and 47% homologous to the *E. Coli* product (8). The response is now generally defined by the increased synthesis of a small number of heat shock proteins with a corresponding decrease in the synthesis of most other cellular proteins.

Brunet and Giacomoni (9) suggested that exposure of tissue to intense light is capable of increasing the synthesis of heat shock proteins. Their study examined the effects of various wavelengths of ultraviolet light (UVA and UVB) on mouse epidermis which simulated the potentially damaging solar irradiation to which humans are exposed daily. UVB (320-360 nm) and UVA (300-400 nm) irradiation increase the amount of an mRNA hybridizing to a DNA probe from the gene of the human hsp70 family of proteins. Brunet and Giacomoni also determined that the response is not caused by an increase in temperature.

UVA light can also induce a smaller molecular weight heat

shock protein, hsp32 (10). This study examined the effect of near UVA (334 and 365 nm) radiation on normal human skin fibroblasts and established that hsp32 is induced after exposure. This study did not, however, indicate whether the energy delivered is of sufficient energy to raise the temperature of the cells.

Sodium arsenite is a very effective inducer of the heat shock response (4, 10-12). This sulfhydryl reagent is thought to bind to cysteine residues of proteins (13). Amino acid analogs (14, 15) will also induce the response. It is thought that the uptake of these analogs produces abnormal proteins, thereby triggering the stress response. It has been suggested that abnormal proteins serve as a trigger for the induction of hsps (16, 17). Other stresses that appear to cause induction of hsps may include the antibiotic puromycin (18, 19), certain prostaglandins (20), ethanol (21), transition series metals such as copper, cadmium, zinc and mercury (22) and even LSD (23, 24). It is believed that antibiotics such as puromycin can inhibit protein synthesis and turnover, resulting in the accumulation of abnormal proteins within the cell. In Santoro's study, prostaglandins with antiproliferative activity (PGA<sub>1</sub> and PGJ<sub>2</sub>) induce hsps, but the exact mechanism of induction is unknown. Transition series metals bind to cysteine residues in a manner similar to arsenite (20). Cosgrove's study on LSD and rabbits found that this addictive drug will induce whole body hyperthermia to such an extent that hsps are induced (23). The mechanism of hsp induction when cells are exposed to ethanol remains unclear.

Some inducers of the stress response are specific for a particular heat shock protein. As mentioned above, the small molecular weight hsp32 is the only stress protein induced when cells are exposed to near UV light (10). Since heat is not a major component of UV irradiation, it is possible that induction of this particular stress protein is wavelength-dependent. Keyse's study utilized incoherent, long wavelength UV light (10). Coherent light, such as that emitted by a laser, is a single wavelength of light traveling unidirectionally and in phase. In contrast, incoherent light such as the UV light used in Keyse's study mentioned above is made up of one or more wavelengths of light all traveling in different directions and out of phase.

Our hypothesis tested whether the strong thermal component associated with the long wavelength coherent light from the CO<sub>2</sub> laser would induce the stress response. Most investigations of hsp synthesis have utilized radiolabeling and gel electrophoresis followed



by autoradiography to detect hsps. With the increased availability of hsp antibodies, the possibility of localizing these proteins immunochemically has become feasible. In our study, we used radiolabeling, autoradiography and immunolocalization with a mouse monoclonal anti-hsp72/73 to determine the time course of hsp production after a brief laser exposure.

## MATERIALS AND METHODS

### Cell Culture

WI38 human diploid fibroblast cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultivated into 75 cm<sup>2</sup> flasks (Costar, Cambridge, MA) and maintained in Dulbecco's Minimum Essential Medium (Sigma Chemical Company, St. Louis, MO) supplemented with 10% fetal bovine serum (Hyclone, Logan, VT). Cultures between passages 20-32 were used for irradiation studies. These cells were subcultivated with trypsin/EDTA into 24-well multiplates (Costar, Cambridge, MA) at a density of 50,000 cells/well. Cells were used at confluence. Two days prior to CO<sub>2</sub> laser radiation, the media was changed to serum-free Fibroblast Growth Media (FGM; Clonetics Corp., San Diego, CA) containing 5 ug/ml of insulin and 1 ng/ml bFGF. This was done to define the system. Serum-containing media contain unknown components which may adversely affect the behavior of the cells. Eliminating the serum erases this concern.

### Laser Irradiation

A carbon dioxide laser (Coherent Radiation Laboratories Model # 41), with a wavelength of 10.6  $\mu\text{m}$ , was used in these studies. Prior to irradiation, the media was removed and replaced with HEPES buffered Hank's Balanced Salt Solution (HBSS, Gibco Laboratories, Grand Island, NY) without phenol red. Immediately before irradiation, the HBSS was removed, then replaced immediately after irradiation. The media was replaced with the buffer when cells were removed for prolonged periods of time (up to 30 min maximum) to maintain pH because the media will not maintain the necessary pH outside of a controlled environment, such as an incubator. Range finding studies conducted in our laboratory indicated that an irradiance of 2.9 W/cm<sup>2</sup> at an exposure duration of 0.8 sec (radiant exposure of 2.32 J/cm<sup>2</sup>) produced the maximal heat shock response. These studies consisted of taking a series of output energies ranging from 1.16 J/cm<sup>2</sup> to 4.64 J/cm<sup>2</sup> and evaluating gels for increased levels of hsp's after exposure of cells to these energies. Higher output energies such as 4.64 J/cm<sup>2</sup> destroyed the cells, whereas lower energies such as 1.16 J/cm<sup>2</sup> were not adequate to induce the hsp's (see Table 1). To ensure that only the cells in the treated well were irradiated during any single exposure, a carbon aperture 14 mm in

diameter was placed directly above the cells cultured in plastic cylindrical wells of 16 mm in diameter. A 14 mm diameter target produced by the carbon dioxide laser on thermal paper was used to align wells for each exposure.

Untreated cells served as negative controls, while sodium arsenite-treated cells served as positive controls. Cells utilized as positive controls were treated with 75  $\mu$ m sodium arsenite in FGM for one hour. After the one hour treatment, sodium arsenite medium was removed and replaced with arsenite-free FGM. All control runs were treated the same as experimental runs in that media was removed and replaced with HBSS prior to exposure of experimental cells to the CO<sub>2</sub> laser.

Exposure Duration (sec)	Irradiance (J/cm <sup>2</sup> )	hsp70 induction
.4	1.16	+
.6	1.74	++
.8	2.32	+++
1.0	2.9	++
1.2	3.48	+
1.4	4.06	-
1.6	4.64	-

Table 1. Summary of range-finding studies. Power output equals 2.9W/cm<sup>2</sup>. Irradiance equals power X exposure duration. + = less than 25% of cells responding to treatment; ++ = 25-75% of cells responding to treatment; +++ = more than 75% of cells responding to treatment; - = no response in cells due to death of cell culture

#### Assessment of Time Course Synthesis of Stress Protein by [<sup>35</sup>S]methionine Incorporation and Polyacrylamide Gel Electrophoresis

The time points chosen for the study of newly synthesized proteins after treatment were 2, 4, 8, 12, 24, and 30 hr. These time points are represented as columns 1, 2, 3, 4, 5 and 6 respectively in a 24-well multiplate (see Figure 2). One hour prior to the designated time point, the medium was removed and replaced with 250  $\mu$ l of methionine-free media containing [<sup>35</sup>S] methionine at 10  $\mu$ Ci/ml for incorporation of label into newly synthesized proteins. After incubation, the radiolabeled media was removed and cells were lysed with 200  $\mu$ l of lysis buffer containing 2% sodium dodecyl sulfate, 10% glycerol, 12.5% 0.5M Tris HCl buffer (pH 6.8) and 0.2 ml of 1%

bromophenol blue in deionized H<sub>2</sub>O. The lysates were collected and stored at -20°C.

Prior to analysis, lysates were thawed to room temperature. Once thawed, 4 µl of 2-mercaptoethanol was added to the 200 µl samples to obtain a total concentration of 2% 2-mercaptoethanol. After heating in boiling water for 5 min to solubilize samples they were centrifuged at 13,000g for 15 min to remove particulates. [<sup>35</sup>S]methionine incorporation was determined by precipitating 5 µl aliquots with an equal volume of ice cold 10% trichloroacetic acid (TCA) on ice for 5 min and spotting onto 0.45-µm nitrocellulose membrane filters. After three washes with ice cold 10% TCA, filters were placed into scintillation vials with 10 ml of Opti-fluor scintillation fluid and counted in a liquid scintillation counter. Total disintegrations per minute were used to adjust the load volumes so that each lane on the polyacrylamide gels contained the same amount of radioactivity, and therefore approximately the same amount of protein.

### Gel Electrophoresis

Samples were analyzed by one dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) according to Laemmli (25). Tetramethylethylenediamine (TEMED), Tris HCl and Tris base were obtained from Sigma Chemical Co. (St. Louis, MO). Acrylamide, glycine, bromophenol blue, and ammonium persulfate were purchased from Bio-Rad Laboratories (Richmond, CA). All other chemicals used were of reagent grade and purchased from standard commercial sources. Running gels contained 10% polyacrylamide in 0.375 M Tris-Base, pH 8.8, with 0.01% SDS. Stacking gels contained 4% acrylamide in 0.5 M Tris-HCl, pH 6.8, with 0.1% SDS. The gel solutions were filtered through 0.45-µm nitrocellulose filters, degassed and chemically polymerized by the addition of TEMED and ammonium persulfate (3.3 ml 10% ammonium persulfate and 0.5 ml TEMED per liter running gel, 3 ml 10% ammonium persulfate and 1 ml TEMED per liter stacking gel). Stacking gels were 2 cm long and running gels were 11.5 cm long. The tank buffer consisted of 25 mM Tris buffer, with 0.1% SDS, and 1.5% glycine. Samples were loaded into gel lanes and a current of 35 mA was used for electrophoresis until the molecular weight marker loaded onto lane one reached the bottom of the gel. Gels were immersed in fixative (25% isopropyl alcohol and 10% glacial acetic acid) and allowed to shake for one hour.

### Fluorography

Fluorography was performed essentially as described by Laskey and Mills (26). Gels were washed in isopropyl alcohol to remove excess water and impregnated with diphenyloxazole (PPO) (4 g PPO per liter of glacial acetic acid, xylene, and ethanol, v/v 55:30:15) then washed twice by soaking in distilled H<sub>2</sub>O for 1 hr. Gels were dried onto filter paper at 60°C for 75 min. (Bio-Rad slab gel dryer, Richmond, CA), then opposed to film (Kodak X-Omat R) in cassettes (regular Kodak X-Omatic). Film was pre-exposed to 0.1 optical density (OD) units using a camera flash with an amber safety filter and filter paper over the lens to diffuse the light. The film was stored at -70°C for the appropriate exposure time and then developed. To quantitatively measure stress-protein levels, fluorograms were scanned with a laser densitometer (LKB Ultrosan XL, Bromma, Sweden). The level of each stress protein was expressed as a percent relative abundance of the total resolved radiolabel.

### Time Course of Hsp70 Immunolocalization

The time points chosen for immunolocalization of newly synthesized hsp70 after treatments were 4, 8, 12, 24, 32 and 48 hr. The untreated cells served as time zero. At each time point, the media was removed and the cells were rinsed twice with Dulbecco's PBS (Sigma Chemical Company, St. Louis, MO). After rinsing, the cells were immediately fixed at room temperature for 5 min in 1:1 ethanol:acetone (-20°C). The fixative was removed and the cells air dried at room temperature. Once the cells were dry, the plate was placed back into the tissue culture incubator. After all cells were fixed at the appropriate time points within the 24-well multiplate, the plate was stored in a dessicator at 4°C before immunocytochemical staining.

Monoclonal antibody to hsp70 was obtained from Oncogene Science, Inc. (Manhasset, NY). Oncogene Science, Inc. reports that their antibody cross reacts with all mammalian cell lines tested by them thus far (murine, rat, rabbit, porcine, bovine and human cell lines).

Prior to immunocytochemical procedures, cells were removed from the dessicator and rehydrated in Dulbecco's PBS containing 0.25% Tween 20 (Sigma Chemical Co., St. Louis, MO) and 0.1%

sodium azide (Aldrich Chemical, Inc., Milwaukee, WI), pH 7.3, for 10 min at room temperature. The immunocytochemical procedure involved the following steps:

- 1) In the blocking step, cells were incubated in 0.5 ml Dulbecco's PBS containing 0.5% casein (Sigma Chemical Company, St. Louis, MO) and 0.05% thimerosal as a preservative (Sigma Chemical Company, St. Louis, MO) for 15 min at room temperature. This blocking reagent, adjusted to pH 7.4, blocks all non-specific binding sites and eliminates all background labeling.
- 2) Excess casein thimerosal buffer was drained from the wells and wells were blotted around the edges to remove excess blocking reagent.
- 3) Cells were incubated for one hr at room temperature and overnight in the cold (4°C) with 250 µl mouse monoclonal antibody to hsp72/73 diluted 1:100 in the casein thimerosal blocking reagent used in step 1. Preliminary trials indicated that this dilution produced the most intense labeling with minimal background staining.
- 4) Cells were drained of excess antisera and washed three times for five min each wash with 2 ml PBS containing 0.25% Tween 20 and 0.1% sodium azide.
- 5) Cells were incubated for one hr at room temperature with 250 µl biotinylated horse anti-mouse antibody. This secondary antibody is commercially bought from Vector Laboratories (Burlingame, California) and is diluted 1:50 in casein thimerosal blocking buffer.
- 6) Cells were washed as in step 4.
- 7.) Cells were incubated for one hr at room temperature with 250 µl gold conjugated streptavidin (Biocell Gold Conjugates, United Kingdom, via Ted Pella Inc., Redding, CA). The conjugated gold/streptavidin complex was diluted 1:50 in casein thimerosal buffer. The size of the gold particles was 5 nm.
- 8) Cells were fixed in 0.5 ml of 2.0% glutaraldehyde in Dulbecco's PBS for 10 min at room temperature. This step stabilized the antibody/antigen complex and therefore prevented loss of antibody/antigen complex during vigorous washing of cells.
- 9) Cells were washed with deionized water three times for 5 min each

wash.

10) Cells were incubated in 1-2 ml silver enhancement solution (Biocell Gold Conjugates, United Kingdom, via Ted Pella Inc., Redding, CA) for 15-30 min or until a desirable staining product was obtained. Reaction was evaluated under a microscope periodically since a Biocell silver enhancement product was used that is relatively insensitive to subdued room lights. The enhancer and initiator solutions were mixed in a 1:1 ratio immediately prior to use and the reaction product appeared as a dark brown to black precipitate when viewed under a light microscope.

11) After reaction was completed, the cells were rinsed in running deionized water for 2-3 min.

12) Cells were incubated in 500  $\mu$ l 0.01% aqueous gold chloride solution (VWR Scientific, San Francisco, CA). This step intensified the dark brown to black precipitate that results from silver enhancing, and allowed for a clearer signal and better quality micrographs.

13) Cells were temporarily mounted with one drop of 10% glycerol in deionized H<sub>2</sub>O and covered with a 13mm coverslip.

The biochemical principle behind this immunochemical procedure is schematically illustrated in Figure 1. This procedure basically utilizes the binding affinity that streptavidin has for biotin in nature. Biotin is a vitamin that belongs to the vitamin B complex family. It is found primarily in yeast, egg yolk and liver. Streptavidin is a protein that is found primarily in bacteria and physiologically is responsible for the inactivation of biotin. It therefore has a high affinity for the biotin molecule. During incubation of the cells in primary antibody, the Fab portion of the antibody binds to the specific antigen, in this case hsp72/73 (See Figure 1). Once this binding of primary antibody to the antigen has occurred, the secondary antibody is added to the wells after washing. This secondary antibody must be directed against the Fc portion of the primary antibody in question. In this case the secondary antibody is directed against the Fc portion of mouse IgG. This secondary antibody is in turn conjugated to biotin. The tertiary structure, the streptavidin-gold complex, will then bind to the biotin on the secondary antibody. Since the 5 nm gold particles are not resolvable under light microscope conditions, a silver enhancement solution must be added to the complex. The silver deposits on the gold such that after several min (15-30 min) a dark

brown to black precipitate becomes visible under the light microscope. After the reaction is complete, the cells are rinsed and a 0.01% aqueous gold chloride solution is added to the wells to intensify the signal. The cells are rinsed, mounted and readied for viewing.

Appropriate controls were provided on separately treated plates. On one plate the primary antibody was omitted. On a second plate the secondary antibody was omitted. The streptavidin step was omitted on a third plate. A fourth plate was run as a control in which a normal pre-immune mouse serum was used in place of the anti-hsp antisera.

Cells were mounted and examined with a Nikon Diaphot inverted microscope. Photographs were made with Kodak TMax 100 black and white film (Eastman Kodak Co., Rochester, NY) and processed by standard techniques.

### Estimation of Temperature Based on Irradiation

To estimate peak temperatures attained by the cells during the brief laser exposure, a Biotherm (Linear Laboratories, Sunnyvale, CA) non-contact infrared thermometer (Model C-600M) was used. The thermometer was calibrated with 100° C water. Cells were plated on 5-cm diameter plastic petri dishes (Falcon, Oxnard, CA) and exposed to laser radiation in a manner similar to that described above. The probe of the thermometer was placed at an angle of 52 degrees from vertical and at a 2.5 cm vertical standoff distance. This takeoff angle prevented detection of the diffusely scattered infrared from the CO<sub>2</sub> laser, as opposed to measuring temperature. In addition, temperature readings were taken at several distances from the source and then extrapolated.

The field of view of the probe projected onto the horizontal plane of the sample was an ellipse with the major axis = 2.3 cm and minor axis = 1.43 cm. The area heated by the laser was a circle 1.5 cm in diameter and was centrally covered by the ellipse. The peak (spatial)  $\Delta T$  was present within the circle, and as a result we have the following relationship:

$$\text{average } \Delta T / \text{area ellipse} = \text{peak } \Delta T / \text{area circle}$$

$$\text{peak } \Delta T = \text{average } \Delta T \times (\text{area circle} / \text{area ellipse})$$

$$\text{area ellipse} / \text{area circle} = (\pi \times (a \times b) / 4) / (\pi \times (d \times d) / 4) = 1.47$$

The mean average  $\Delta T$  for our ambient experimental conditions (2.9 W/cm<sup>2</sup>/0.8 sec) as measured by IR thermometry was 45.4° C (n=18 measurements). The standard deviation of the data equaled 2.2.

Therefore, the mean peak  $\Delta T$  was 67° C at room temperature. This



**GOLD W/ SILVER ENHANCEMENT IMMUNO. TECHNIQUE**

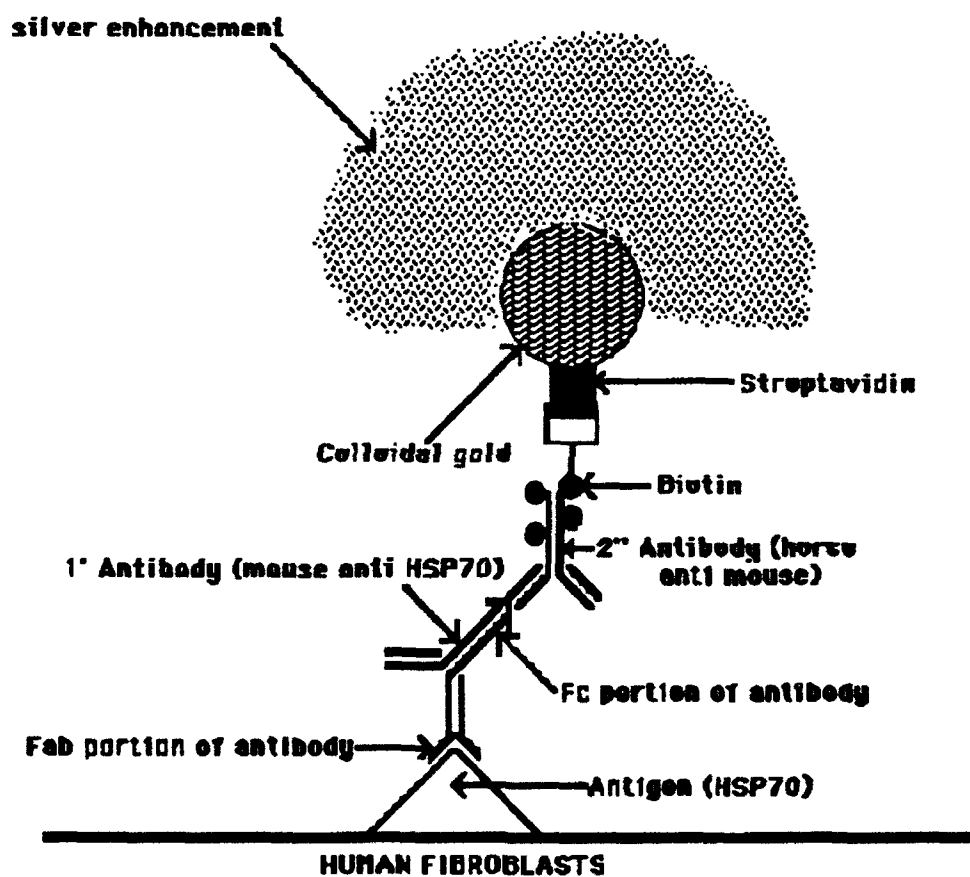


Figure 1. Schematic diagram showing biotin-streptavidin immunolabeling with silver enhancement.

was expressed by the following relationship:

$$\text{area ellipse/area circle} \times \text{actual measurement} = \Delta T$$

$$1.47 \times 45.4 = 67^{\circ} \text{C.}$$

#### Use of Hot Media Pulses

To ascertain if the estimated increase in temperature was due to CO<sub>2</sub> laser irradiation, fresh serum free Fibroblast Growth Media (FGM) was preheated to 70°C. Serum-free media do not contain any components which would otherwise be sensitive to heat as high as 70°C, therefore the media were not altered by the temperature rise, eliminating all possibilities of altered media causing induction of hsps. The media in the multiplate were removed and immediately replaced with the 70°C media for 1-2 sec. The hot media were then removed, discarded and immediately replaced with fresh FGM. The fresh media which, were initially heated to 70°C, were then allowed to cool to 65°C, and the procedure was repeated in the next row of cells. This procedure was repeated for 60°, 55° and 50°C temperatures. The temperature was measured by insertion of a BAT-10 thermometer (Physitemp, Clifton, NJ) inside the culture tube containing the medium. Control medium was maintained at 37°C. Once cells were treated with the hot media, they were placed back inside the incubator for 8 hr to simulate the 8 hr time point of the laser-treated cells. Cells were then fixed and processed for immunocytochemistry as described above.

## RESULTS

### Heat shock protein induction

The results of exposure of human fibroblasts to CO<sub>2</sub> laser and arsenite as a function of time after radiolabeling and gel electrophoresis are illustrated in Figure 2. The negative control treatment (lanes 1-6) shows bands corresponding to stress proteins with approximate molecular weights of 70kD and 32kD. For the six time points used (2, 4, 8, 12, 24, and 30 hr) for untreated cells, levels of hsp70 and hsp32 remain at low baseline levels. Laser-treated time points (lanes 7-12) revealed an increase in newly synthesized hsp70 at 2 hr when compared to control, with this increase becoming more apparent at 4, 8 and 12 hr. By 30 hr, hsp70 synthesis had returned to baseline levels. Hsp32 was not induced by CO<sub>2</sub> laser radiation, which differed from the response reported by Keyse with respect to ultraviolet radiation (10). Sodium arsenite treatment (75  $\mu$ m for one hour) (lanes 13-18) also revealed an increase in newly synthesized heat shock proteins when compared to control times. This increase in hsp appeared to peak at 4-8 hr, with a return to baseline levels by 30 hr. Sodium arsenite induced hsp32 as indicated by fluorography, with increased levels of hsp32 present throughout the six time points.

<sup>35</sup>S-labeled proteins in the fluorograms were scanned with a densitometer to quantify hsp70 protein levels between treatments. The time course of <sup>35</sup>S methionine incorporation above negative control levels in response to the two treatments is shown in Figure 3. The laser-treated cells revealed an increase in newly synthesized hsp70 at 2 hr. Methionine incorporation peaked at 4 hr, with subsequent decreases in newly synthesized hsp70 at 8, 12, and 24 hr. Hsp70 levels returned to baseline values by 30 hr. Sodium arsenite-treated cells also showed a peak in newly synthesized proteins at 4 hr, but not as much as in laser-treated cells. Arsenite-treated cells also showed a stepwise decrease in hsp 70 levels up to 24 hr with a second peak in newly synthesized proteins evident at 30 hr.

### Immunolocalization of Hsp72/73 in Human Fibroblasts

The end product of the biotin/streptavidin/gold detection procedure is a black precipitate. A macroscopic view of the labeling within the 24 well-multiplate is shown in Figure 4. The negative control cells revealed virtually no labeling of hsp72/73 through all six

time points studied. The laser-treated cells exhibited a markedly different pattern. By 4 hr, labeling was present within the well. The most intense labeling occurred at 8-12 hr and decreased continuously for 48 hr. The arsenite-treated cells also exhibited intense labeling at 4 hr, with labeling remaining at elevated levels for a full 48 hr.

The microscopic view of cells is shown in Figures 5-10. At 4 hr after induction, intense labeling was evident within the nuclei of both the laser-treated cells and the arsenite-treated cells (Figure 5). Untreated cells revealed virtually no labeling. Negative control cells for this time point and subsequent time points revealed no immunolocalization of hsp72/73 above baseline levels. Included in each figure is a micrograph of the control cells by phase contrast. To avoid interfering with immunolabeling, no histological counterstain was used so phase contrast microscopy was necessary to enhance visualization of control cells.

By 8 hr post induction, the laser-treated cells continued to exhibit intense labeling of hsp72/73 within nuclei (Figure 6). The arsenite-treated cells, however, no longer exhibited nuclear labeling of hsp72/73. Virtually all of the labeling was localized to the cytoplasm of these cells.

By 12 hr post induction, the laser-treated cells exhibited a localization of hsp72/73 in the cytoplasm (Figure 7). The laser-treated cells continued to exhibit immunolocalization of hsp72/73 to the cytoplasm by 24 and 32 hr (Figure 8 and 9). By 48 hr, the labeling within the laser-treated cells had subsided, indicating decreased hsp72/73 within the cell (Figure 10).

The arsenite-treated cells at 12 hr also continued to show increased localization of hsp72/73 to the cytoplasm (Figure 7). The same was true for arsenite-treated cells at 24 and 32 hr (Figure 8 and 9). By 48 hr, the arsenite-treated cells continued to show intense labeling of hsp72/73, as opposed to laser-treated cells, which exhibited a reduction in labeling at this time point (Figure 10). Arsenite is a strong inducer of hsp70, and probably continued to bind to cysteine residues of proteins, resulting in a prolonged stress response.

#### Simulation of Thermal Effects of Carbon Dioxide Laser

A 24-well multi-plate immunolabeled with antibody to hsp72/73 after treatment with pulses of hot media is shown in Figure 11. The multi-plate is set up such that the first column serves as the control

lane (37°C). The second lane contains the cells that were treated for 1-2 sec with media heated to 70°C. Subsequent lanes contained cells treated with media heated to 65°, 60°, 55° and 50°C respectively. The bottom row (D) contained no cells and was a blank. This plate revealed that cells exposed to high temperatures for just a few seconds can survive and produce hsps. The most intense labeling occurred at 70°C, with a decrease in intensity of labeling at 65°C.

Microscopic evaluation revealed that hsp72/73 was localized primarily in the cytoplasm (Figures 12-13). The most intense labeling occurred in the cells treated with 70°C media, with a decrease in labeling exhibited at 65°C and 60°C. Virtually no labeling occurred in control cells (37°C) or in the cells treated with 55° and 50°C medium.

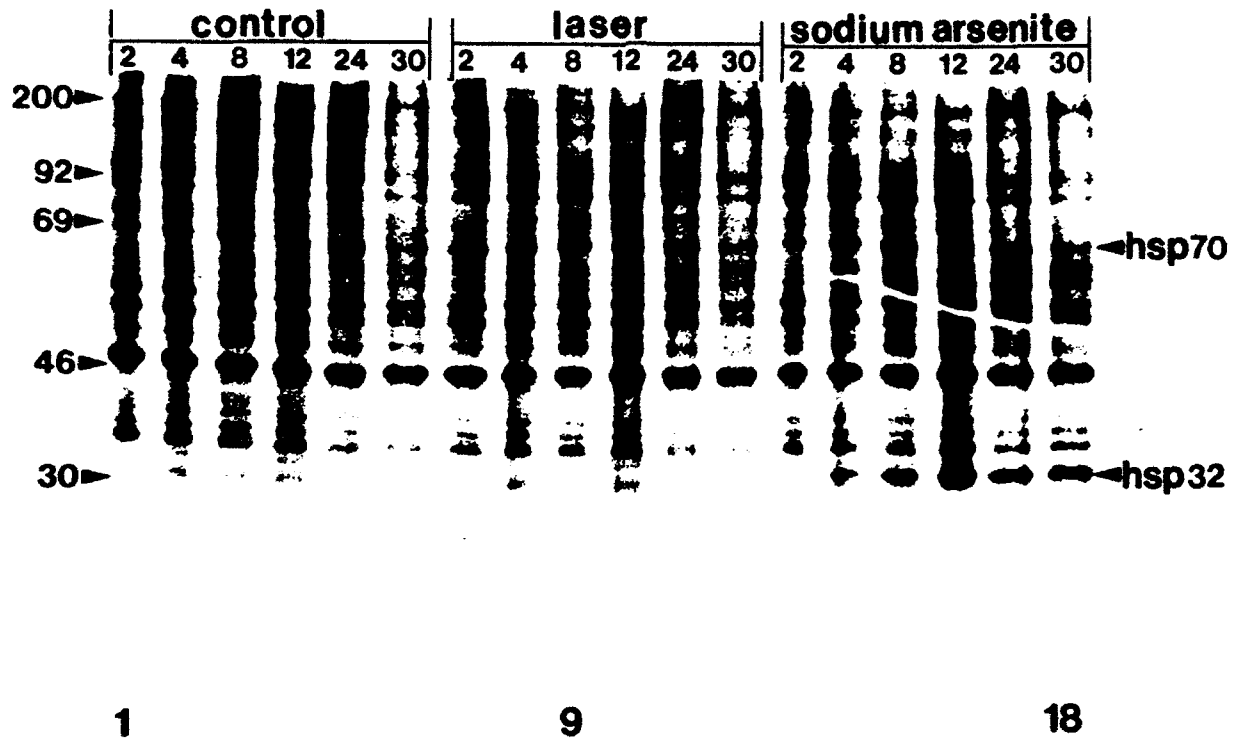
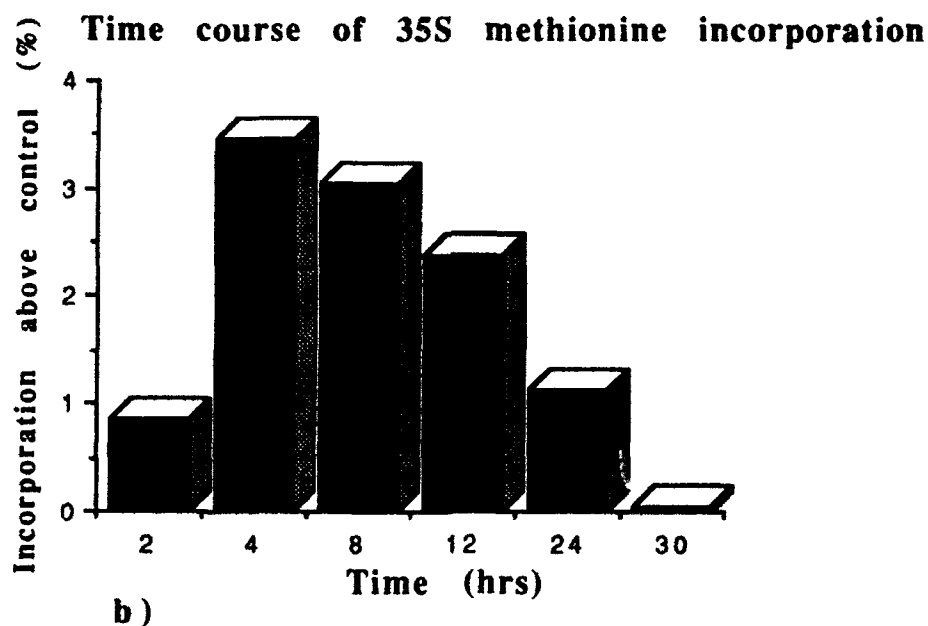
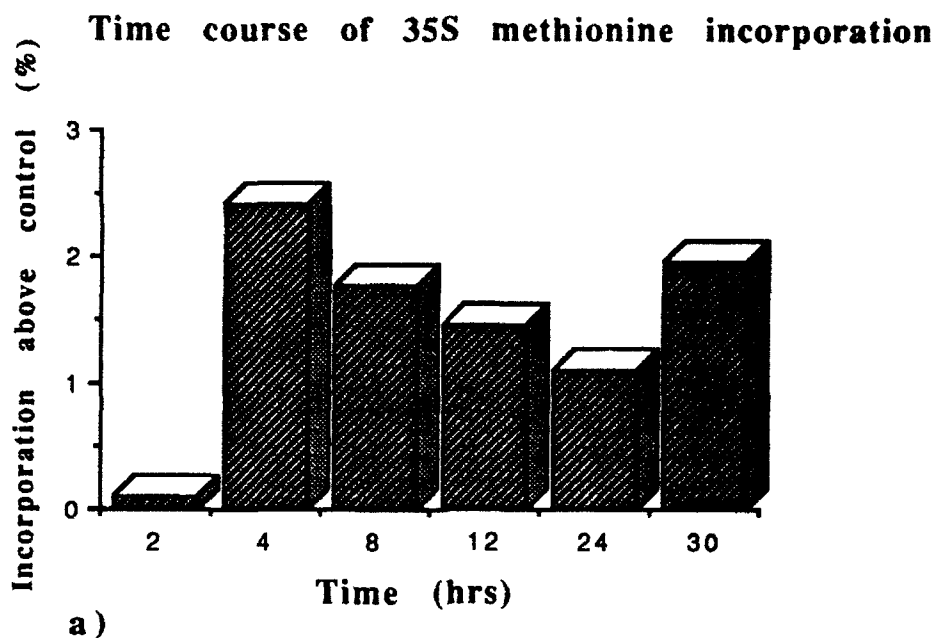


Figure 2. Fluorogram of one-dimensional polyacrylamide gel of stress proteins in human lung fibroblast cell cultures. Cells were exposed to CO<sub>2</sub> laser, sodium arsenite or untreated (Control). Protein samples were prepared as described in materials and methods. Lanes 1-6, control cells; lanes 7-12, laser-treated cells; lanes 13-18, arsenite-treated cells (75  $\mu$ m for 1 hr). Numbers at top of lanes indicate number of hr after induction of hsps.



**Figure 3.** a) Percent relative abundance of the 70kD heat shock protein over time in cells exposed to 75 $\mu$ m sodium arsenite for one hour. b) Percent relative abundance of the 70 kD protein over time in cells exposed for 0.8 sec to a CO<sub>2</sub> laser.

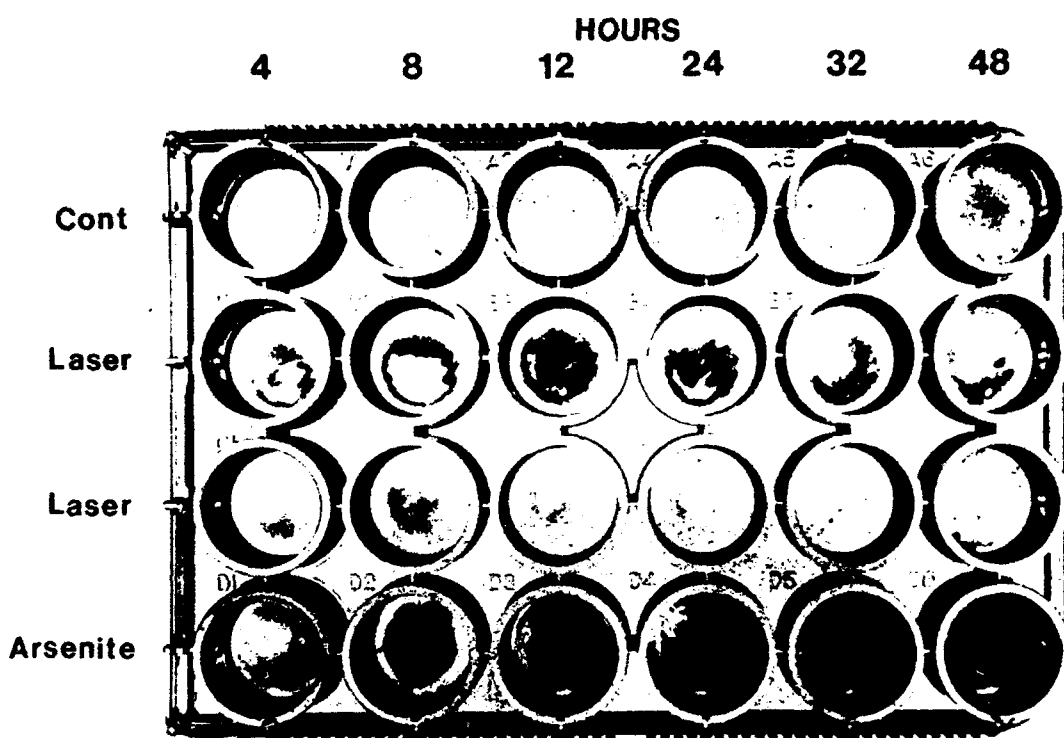


Figure 4. Macroscopic view of 24-well multiplate after immunocytochemical labeling. Top row, untreated control cells; second and third rows, laser-treated cells; fourth row, arsenite-treated cells. Cells were labeled according to methods described in Materials and Methods. Columns represent number of hr after induction.



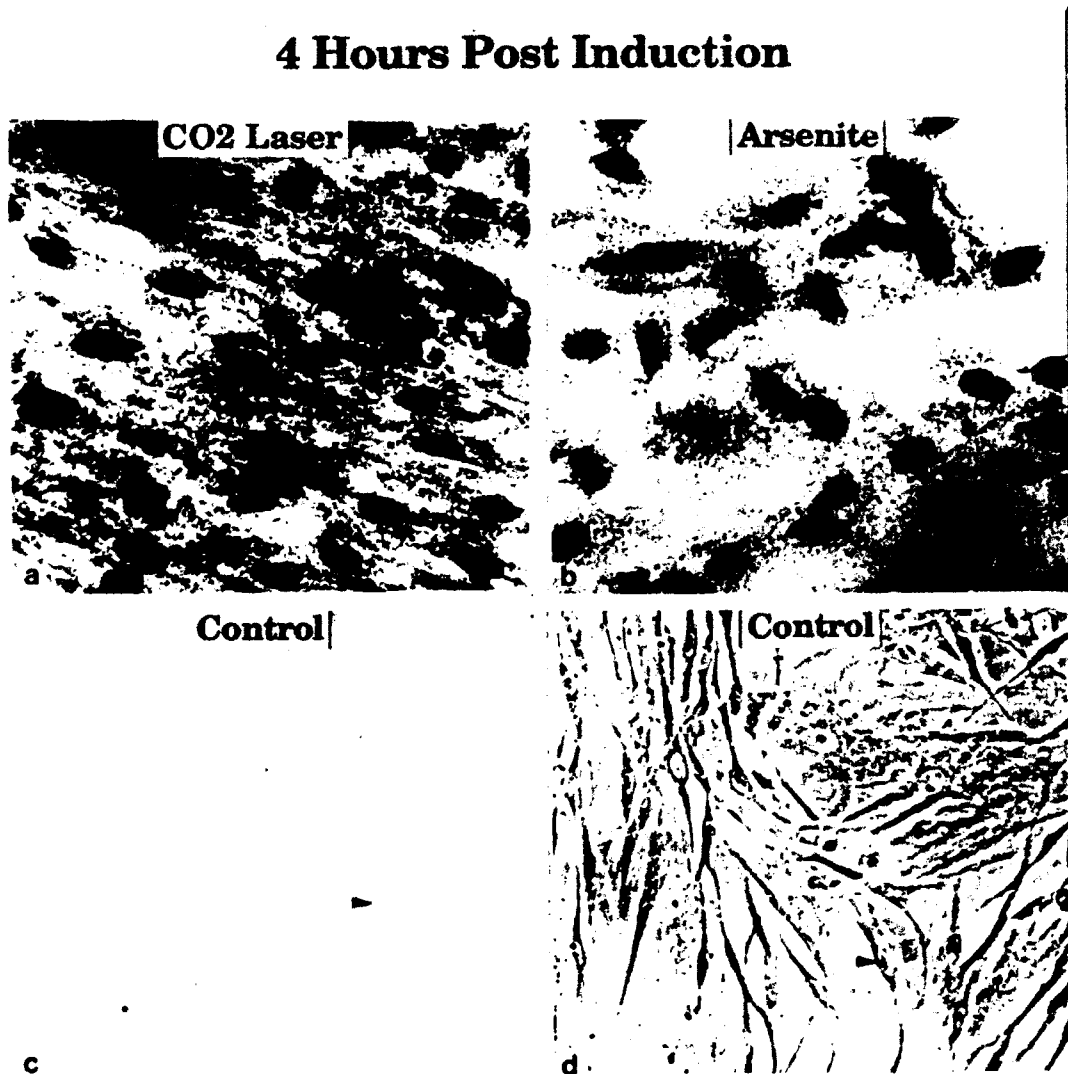


Figure 5. a) 0.8 sec laser exposure at 4 hr after induction; cells indicate labeling of hsp72/73 using monoclonal antibody. b) 1 hr treatment with 75  $\mu$ m sodium arsenite at 4 hr after treatment. c) untreated cells at 4 hr. d) phase contrast micrograph of untreated cells. Magnification equals 2000X.

### 8 Hours Post Induction

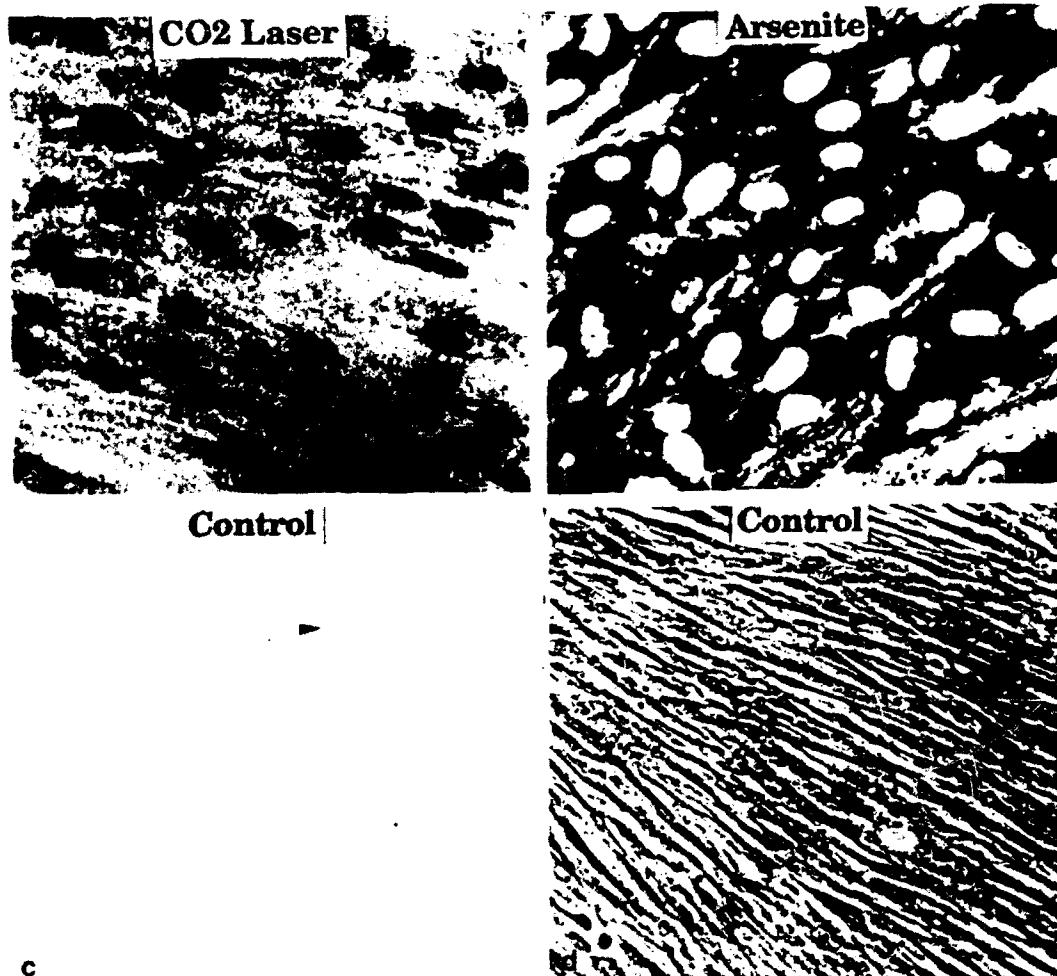


Figure 6. a) 0.8 sec laser exposure at 8 hr after induction; cells indicate labeling of hsp72/73 using monoclonal antibody. b) 1 hr treatment with 75  $\mu$ m sodium arsenite at 8 hr after treatment. c) untreated cells at 8 hr. d) phase contrast micrograph of untreated cells. Magnification equals 2000X.

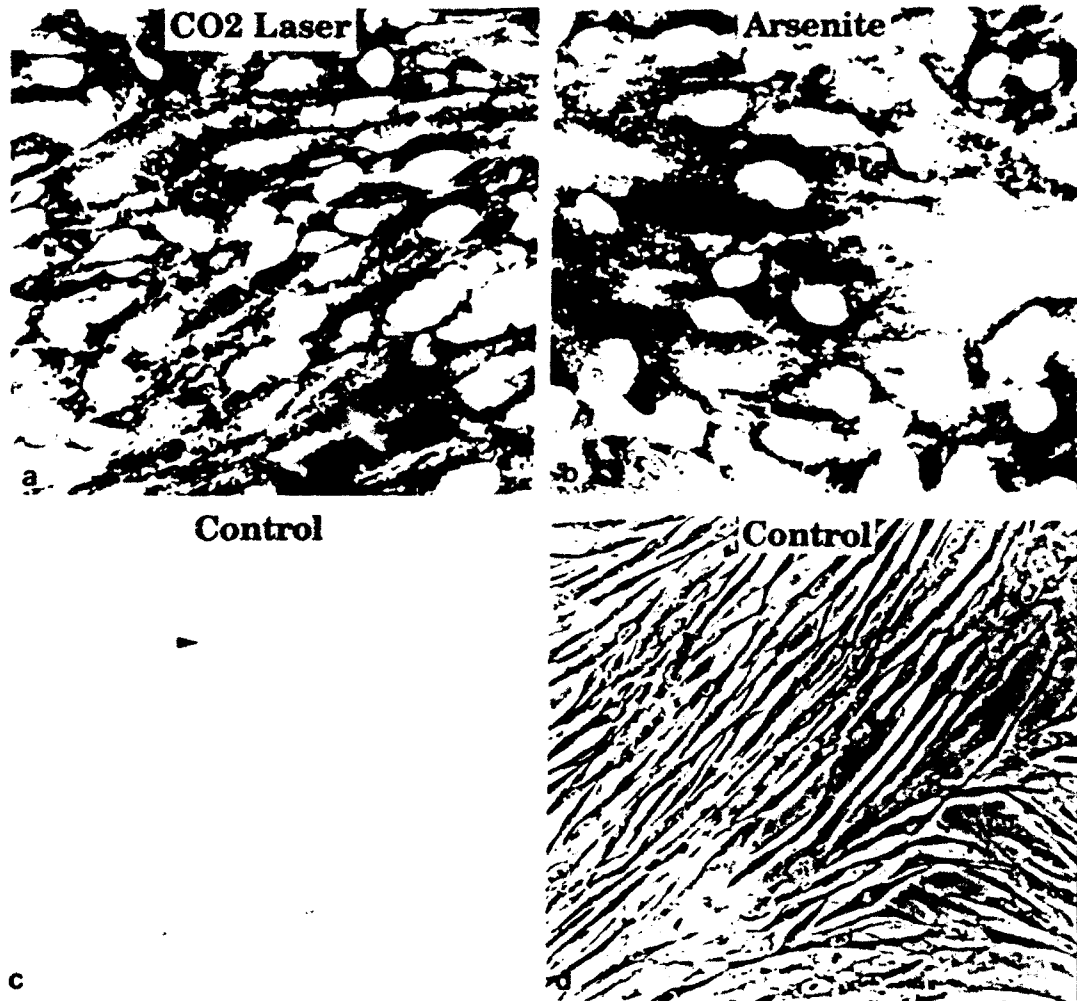


Figure 7. a) 0.8 sec laser exposure at 12 hr after induction; cells indicate labeling of hsp72/73 using monoclonal antibody. b) 1 hr treatment with 75  $\mu$ m sodium arsenite at 12 hr after treatment. c) untreated cells at 12 hr. d) phase contrast micrograph of untreated cells. Magnification equals 2000X.

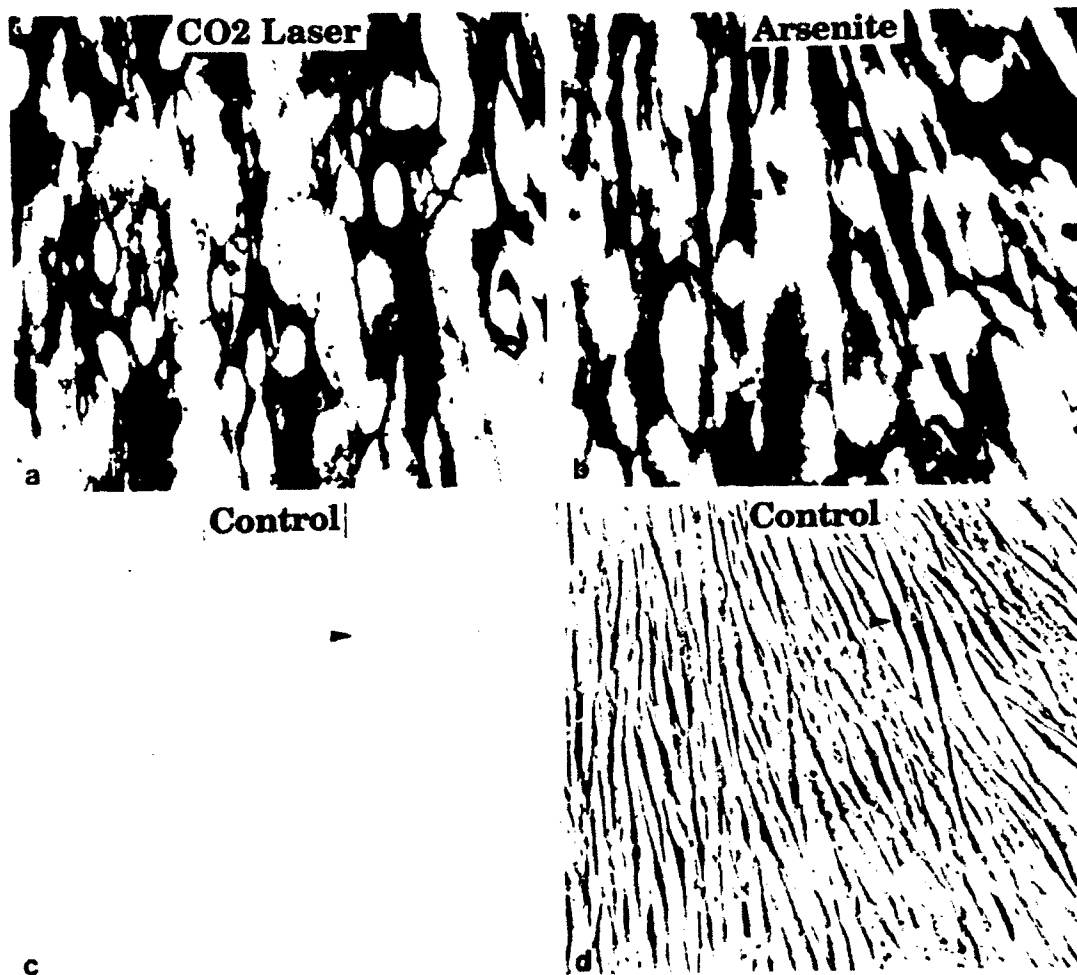


Figure 8. a) 0.8 sec laser exposure at 24 hr after induction; cells indicate labeling of hsp72/73 using monoclonal antibody. b) 1 hr treatment with 75  $\mu$ m sodium arsenite at 24 hr after treatment. c) untreated cells at 24 hr. d) phase contrast micrograph of untreated cells. Magnification equals 2000X.

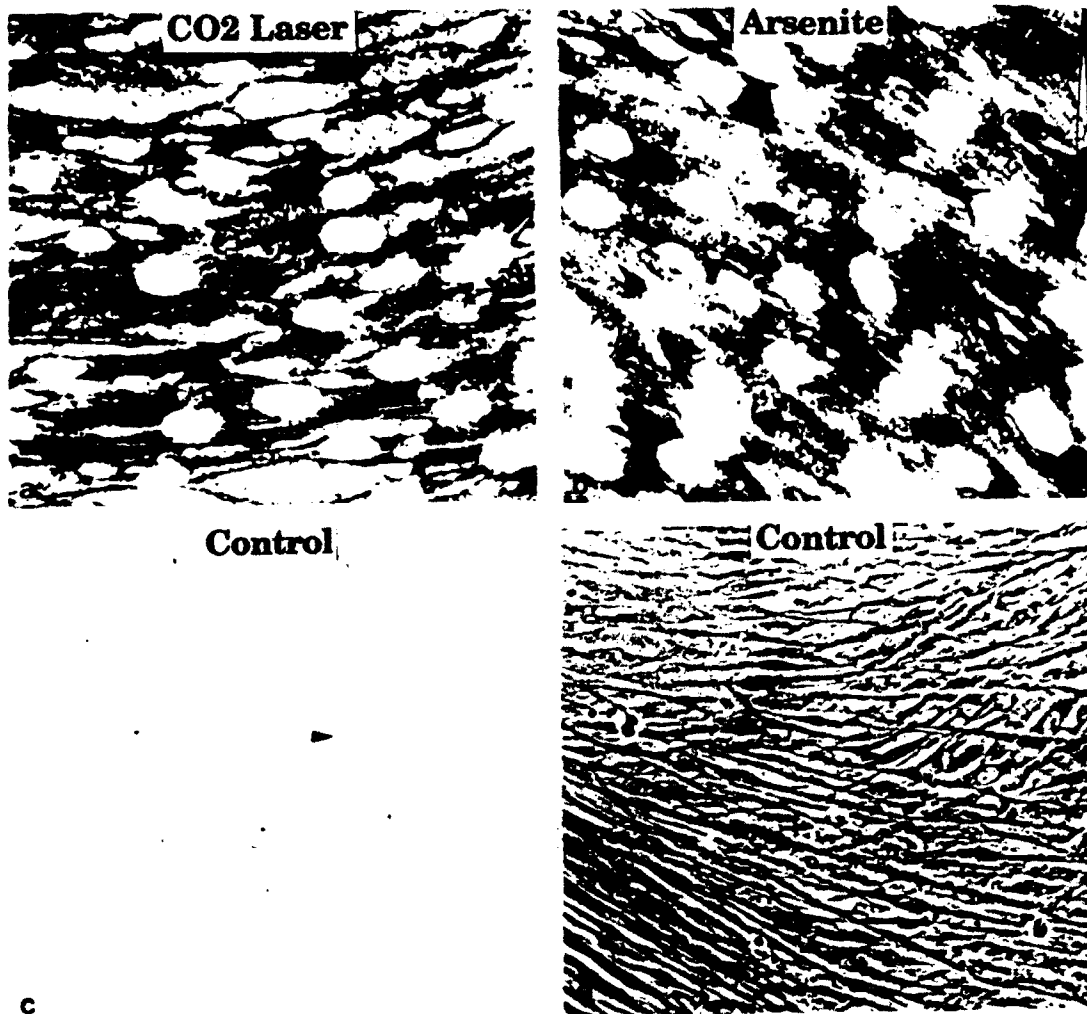


Figure 9. a) 0.8 sec laser exposure at 32 hr after induction; cells indicate labeling of hsp72/73 using monoclonal antibody. b) 1 hr treatment with 75  $\mu$ m sodium arsenite at 32 hr after treatment. c) untreated cells at 32 hr. d) phase contrast micrograph of untreated cells. Magnification equals 2000X.

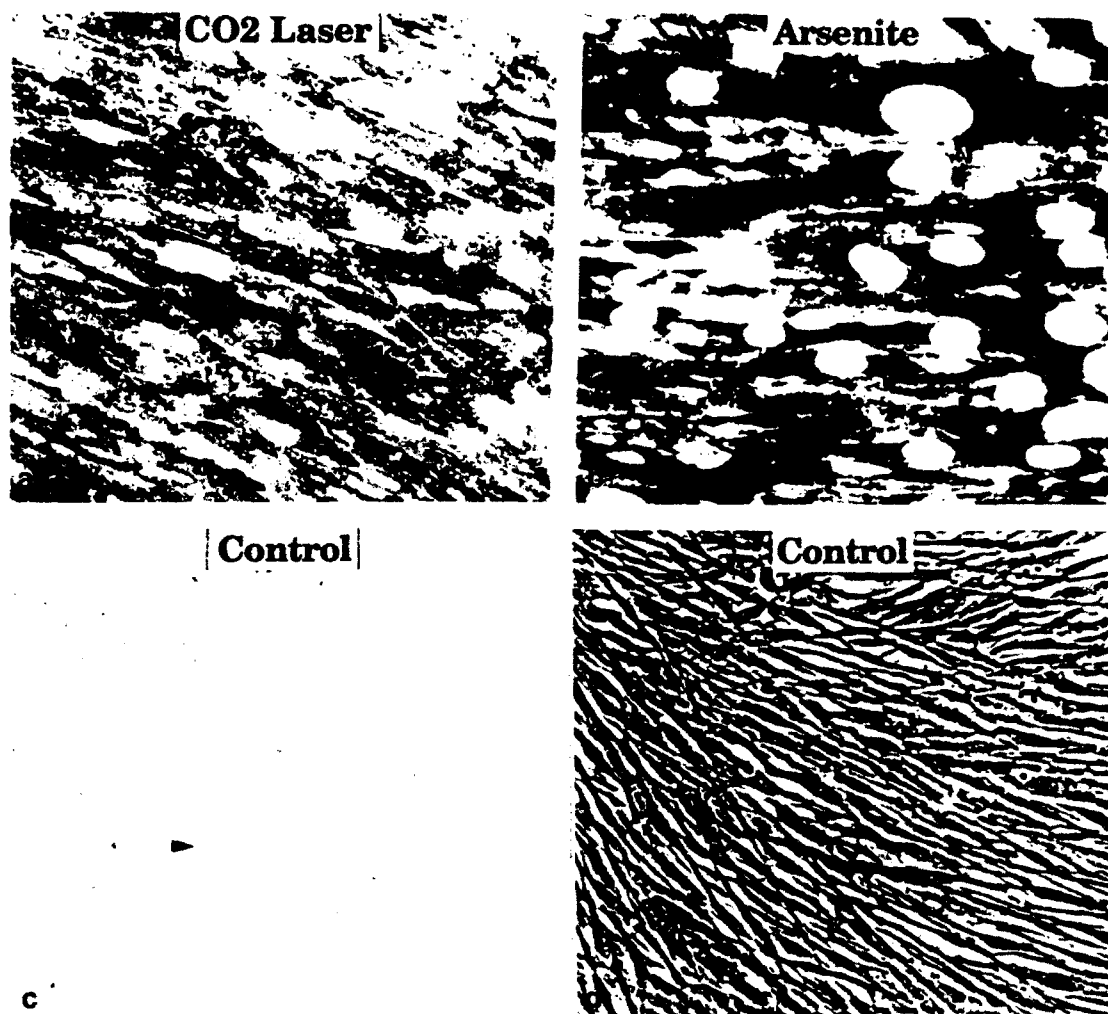


Figure 10. a) 0.8 sec laser exposure at 48 hr after induction; cells indicate labeling of hsp72/73 using monoclonal antibody. b) 1 hr treatment with 75  $\mu$ m sodium arsenite at 48 hr after treatment. c) untreated cells at 48 hr. d) phase contrast micrograph of untreated cells. Magnification equals 2000X.

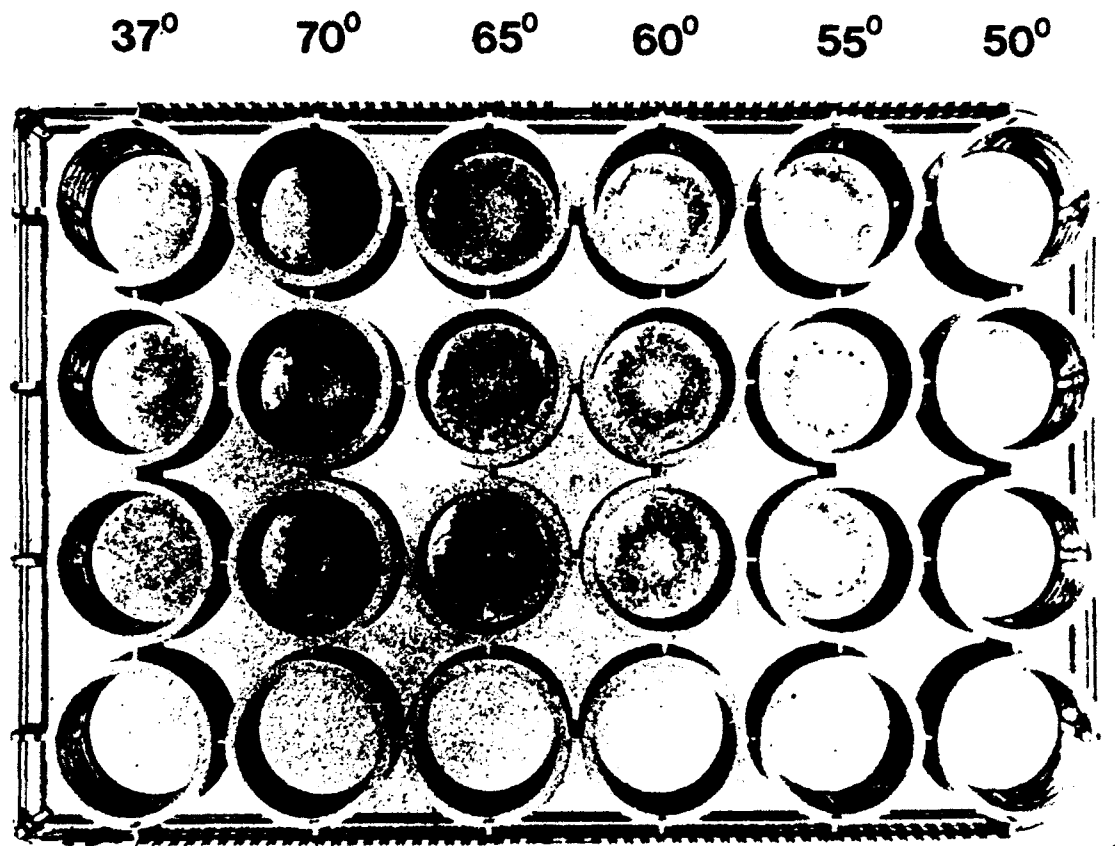


Figure 11. Macroscopic view of 24-well multiplate treated with brief pulses of hot media prior to immuncytochemical labeling. Columns represent temperatures used. Plate was incubated for 8 hr after induction of the stress response. Bottom row is a blank row in which cells were not subcultivated.

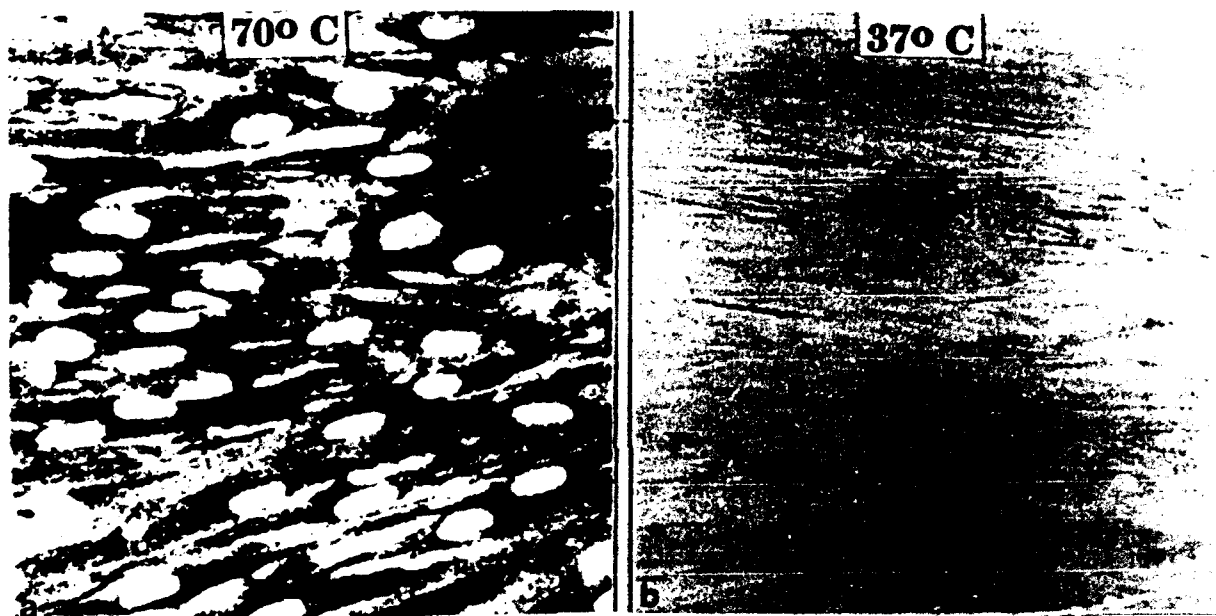
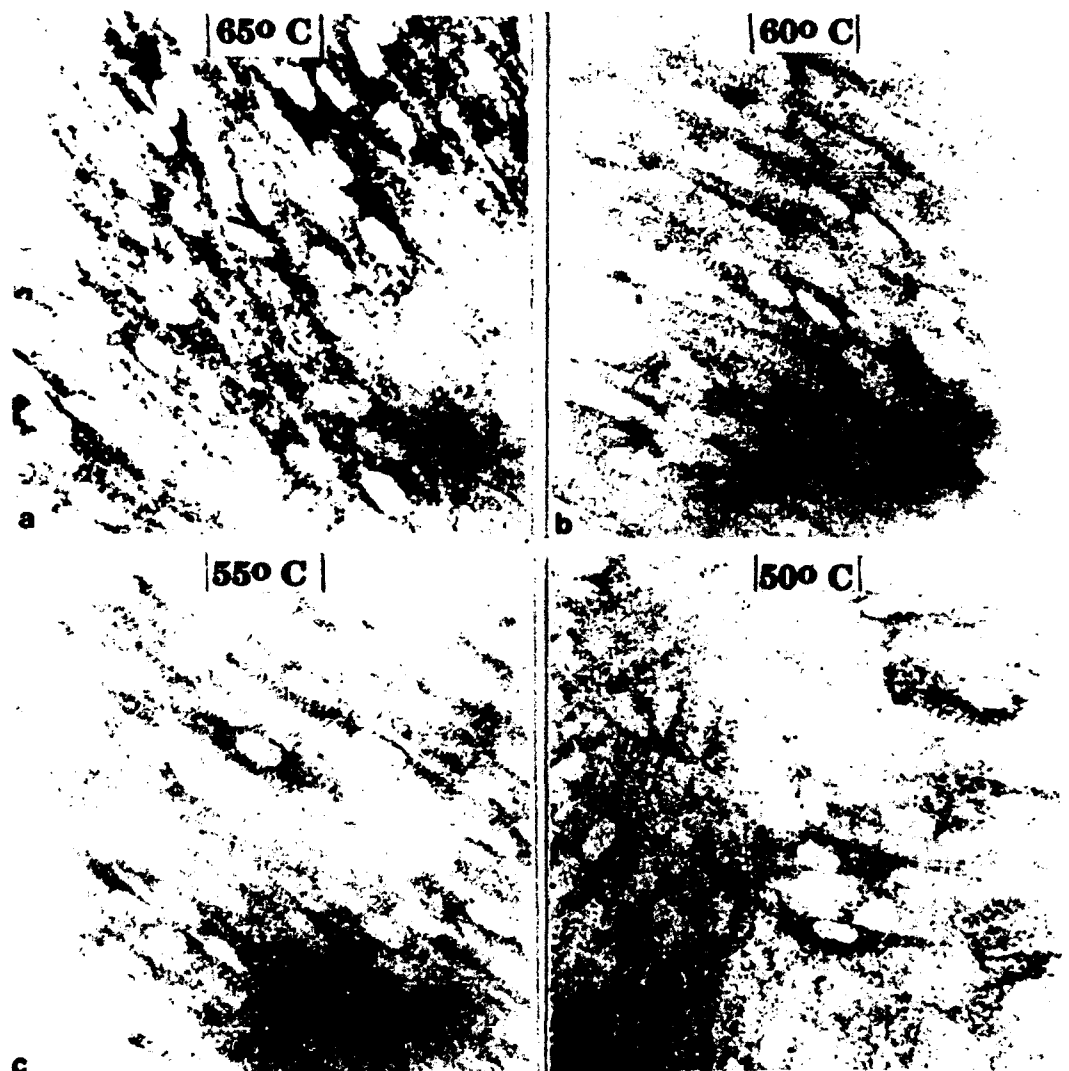


Figure 12. Micrographs exhibiting immunocytochemical labeling of cells after treatment with hot media for 1-2 sec. Cells were allowed to incubate for 8 hr after induction of the stress response. A, 70°C media; B, 37°C media (control). Magnification equals 2000X.





**Figure 13.** Micrographs exhibiting immunocytochemical labeling of cells after treatments for 1-2 sec with hot media of varying temperatures. A, 65°C media; B, 60°C media; C, 55°C media; D, 50°C medium. Magnification equals 2000X

## DISCUSSION

### Background Information

The hypothesis investigated by this study is: do human cells, when exposed to millisecond (.8 sec) pulses from a CO<sub>2</sub> laser, express heat shock proteins. A previous study conducted by Polla and Anderson investigated the possibility of heat shock protein induction utilizing short pulses (2  $\mu$ sec) from a carbon dioxide laser (27). Polla and Anderson discovered that CO<sub>2</sub> laser radiation in fact did not induce heat shock protein synthesis. Although the amount of energy emitted by the laser was in the range of 0-0.8 J/cm<sup>2</sup>, higher energy outputs of 1.3 J/cm<sup>2</sup> in which cell viability was compromised also showed no evidence of hsp synthesis. Although the induction of heat shock proteins has been frequently studied with heat, heavy metals and amino acid analogs, much less has been done with induction by light. Incandescent light is incoherent and is made up of different wavelengths traveling in different directions, all out of phase. Laser (an acronym for light amplification by stimulated emission of radiation) is coherent, i.e., the light is composed of a single wavelength traveling unidirectionally and in phase.

Incoherent near ultraviolet light (300-400 nm) has been shown to induce heat shock proteins in human fibroblasts (10). Since laser light is coherent and more efficient in affecting biological systems, exposure of cells in culture may increase the production of heat shock proteins. One reason why lasers are more efficient is their wavelength or chromatic purity. Depending on the wavelength of laser light used when cells are exposed, certain components of the cell will be highly efficient absorbers of the energy. The molecules that are efficient absorbers of this energy may become altered, resulting in further alteration to cellular function. Cells that are sufficiently stressed may respond by inducing synthesis of hsps.

Another way that laser light can cause induction of heat shock proteins is through the thermal effects of absorption. When tissues absorb light, energy is dissipated as heat. Under appropriate conditions, the temperature rise may be sublethal but sufficient to denature proteins such that induction of hsps occurs.

The results of this study demonstrate that the synthesis of hsps was induced in human cells by at least 800 millisecond exposures to a carbon dioxide laser. This response was selective for hsp72/73.

Although wavelength properties of the CO<sub>2</sub> laser might influence the response, elevated hsp72/73 were duplicated with high temperature and a thermal effect probably explains the presented results. The response described here indicates that much higher temperatures can be used for short periods.

The heat shock proteins are ubiquitous polypeptides whose synthesis is increased in response to adverse conditions. This response has been noted for all cells and organisms studied thus far, and appears to be a basic cellular response to stress. However, the exact role of heat shock proteins in response to stress is not known.

Name	Function	Reference
hsp 28	organization of cytoskeleton and nucleus	10, 28, 29
hsp 32	protection from oxidative stress	10
hsp 56	glucocorticoid receptor function	30
hsp72/73	translocation of proteins across membranes; ATP unfoldase activity	31, 32
hsp90	cytoplasmic carrier of precursor proteins; "chaperone"; normal functioning of steroid receptors	33, 34
hsp110	rRNA transcription	35

Table 2: Summary of heat shock protein functions.

The series of proteins that make up the classic heat shock protein family found in mammalian cells consists of proteins with molecular weights of 28, 32, 56, 72/73, 90 and 110kD. These proteins are induced by stresses such as temperature shock, certain chemicals, and other environmental stresses. Although the heat shock proteins of interest in this study are hsp72 and 73, a brief

summary of functions for the other members of the hsp family is summarized in Table 2.

The function of the small molecular weight stress protein hsp28 is not clear. Various studies have suggested that it is associated with the cytoskeleton during the early stages of the stress response (28, 29). As hsp28 associates itself with the cytoskeleton, it localizes within the nucleus and eventually moves out into the cytoplasm during recovery (36, 37).

Hsp32 is a unique stress protein induced after exposure to near UV radiation and the oxidizing agent hydrogen peroxide (10). This study suggests that hsp32 may be involved in protecting the cell from oxidative stress, which is believed to be the major route of damage to cells when exposed to UV light and especially to H<sub>2</sub>O<sub>2</sub>. Recently, it was suggested that the major 32kD stress protein is in fact heme oxygenase (38, 39). Heme oxygenase is induced by oxidative stress such as H<sub>2</sub>O<sub>2</sub> and UV light, and the cloning of cDNA from induced mRNA populations has allowed these researchers to identify the 32kD protein as heme oxygenase. Sodium arsenite is also a good inducer of hsp32 (38).

Hsp56 is a relatively novel heat shock protein that is thought to interact with glucocorticoid receptors and which forms a complex with hsp70 and hsp90 (30).

The heat shock proteins of interest in this study are hsp72/73, which are the best understood of all the stress proteins. In general, the 73kD protein is present in all cells under normal conditions, and therefore is termed the "constitutive" hsp73kD protein (7). The 72kD protein, on the other hand, occurs only after induction of the stress response and in most cases represents the major translational activity of the induced response of cells, and is therefore termed the inducible hsp72kD protein. Following heat shock treatment, increased synthesis is observed with hsp72 and, to a lesser extent, with hsp73 (40). Even though the 72 and 73kD stress proteins appear closely related, they represent distinct gene products (6, 41).

Under normal conditions, the biochemical role for hsp72/73 is thought to be the translocation of secretory and mitochondrial precursor proteins from their site of synthesis in the cytoplasm across the endoplasmic reticulum and mitochondrial membranes, respectively (31). Precursor proteins in transit through the mitochondrial matrix interact with hsp72/73 at specific sites within mitochondria (32). Hsp72/73 is required within the mitochondrial

matrix for transport across the membrane (42). Under stress, however, much of the cytoplasmic hsp72 is closely associated with ribosomes, and is thought to be important to the functioning of the translational machinery during and after recovery from the heat shock treatment (43). It has also been determined that hsp72/73 is involved in the recovery of nuclear morphology during recovery from heat shock (44). In our study it was observed that hsp72/73 is localized to the nucleus during the first few hours of the response. This occurs as nuclear morphology is changing. Hsp72/73 is most likely involved in the reorganization of the nucleus once induction has occurred after exposure to the CO<sub>2</sub> laser.

Although hsp90 has been studied extensively, its exact function is yet to be determined (45). It has been suggested that one function of hsp90 may be to act as a cytoplasmic carrier of precursor proteins that are destined for either delivery to organelles or associated with intracellular membranes (33). For this reason hsp90 has been designated "chaperonin" (46). It has also been proposed that hsp90 is involved in the functioning of steroid receptors (34). Hormone-free glucocorticoid receptors have been shown to form a complex with hsp90 (as well as hsp70) and are thought to depend on hsp90 for normal expression in cells (47). In addition to its association with steroid receptors, hsp90 has been implicated in protecting mammalian cells from thermal stress (48).

Of all the major members of the heat shock protein family, the least is known about hsp110. This stress protein appears to be associated within the region of the nucleolus that is involved in rRNA transcription <sup>35</sup>.

Although the genetic sequences of the heat shock proteins have been determined <sup>49</sup>, the biochemical events leading to the induction of the heat shock proteins are now being defined. It was suggested that increased activation of a heat shock transcription factor (HSTF) by stresses such as hypoxia or heat shock may lead to the increased transcription of various heat shock proteins <sup>50, 51</sup>. This trans-acting HSTF is thought to be a protein that when activated binds to a cis-acting domain on the DNA molecule. This cis-acting region is termed the heat shock element (HSE), and is activated only under stress when bound by the HSTF <sup>52, 53</sup>.

Under normal growth conditions, heat shock proteins bind to and repress HSTF activities <sup>54</sup>. Heat shock causes the dissociation of

heat shock factor-hsp complexes which then allows for the HSTF to bind to the heat shock element on the DNA molecule. Hsp mRNA synthesis proceeds until hsp levels are high enough so that reassociation of hsps and heat shock factor reestablishes the repressed state <sup>54</sup>.

Although the hsps are present at much higher levels during periods of stress, they are also present in unstressed cells but at much lower levels. Therefore hsps are believed to be essential for cellular survival. For example, it has recently been suggested that stress proteins may play an important role in the immune response because they may be involved with antigen presentation and recognition <sup>55</sup>.

In addition to its role in the folding and unfolding of polypeptides during protein synthesis, the 70-kD hsp appears to be absolutely essential for the normal growth of cells <sup>56</sup>. This study linked a member of the 70-kD heat shock protein multigene family of *Saccharomyces cerevisiae* to growth. Mutants were constructed and substituted in place of wild-type alleles. Mutants lacking the wild-type gene (SSC1) were able to germinate but ceased growth after several cell divisions, suggesting that hsp70 may be involved in the normal growth patterns of cells. Hsp70 is also elevated during various stages of the cell cycle <sup>57</sup>. Hsp70 levels increase 10-15 fold upon entry into S phase and decline by late S and G2. The exact role for hsp70 during this increase in synthesis is incompletely understood. Stress protein genes have also been shown to be activated by hormonal stimulation, such as in embryonic development <sup>58</sup>. These functions suggest that stress proteins may function in situations not related to stress.

It has been observed that induction of hsps is correlated with protection against a secondary lethal exposure to the same and/or different inducing agent. This phenomenon is commonly known as thermotolerance. In 1987 it was discovered that pretreating Chinese hamster ovary (CHO) cells in vitro for 15 min at 43°C enhanced survival of the cells at subsequent temperatures of 45°C <sup>59</sup>. Thermotolerance in response to heat has also been observed in human keratinocytes <sup>60</sup> and *Salmonella typhimurium* <sup>61</sup>. Induction of the heat shock proteins has also been shown to protect various transformed cell lines from lysis by tumor necrosis factor <sup>62</sup>.

In addition to the use of heat as a pretreatment regimen for induction of thermotolerance, metabolic inhibitors have also been

used as a pretreating agent for the purpose of inducing the hsp's and protecting against subsequent lethal exposures to heat. A study conducted in 1982 discovered that treating Chinese hamster ovary cells (CHO) with the protein synthesis inhibitor cycloheximide 2 hr prior to heat stress (45°C for 10 min) protected cells by a factor of 1.8 (when compared to controls) when exposed to the heat challenge <sup>63</sup>. Treatment of CHO cells to other metabolic inhibitors such as puromycin also produced similar results <sup>64</sup>. How increased transcription of the hsp's occurs in the presence of protein synthesis inhibitors is not discussed in the studies cited. It is quite possible that since protein turnover is affected, denatured proteins may accumulate within the cell, thereby sufficiently stressing the cell so as to induce the synthesis of heat shock proteins. The relationship between protein synthesis inhibition and induction of thermotolerance is certainly not limited to CHO cells. This phenomenon has also been observed in *Tetrahymena thermophila* <sup>65</sup> and rat fibroblasts <sup>66</sup>.

Although thermotolerance has been well documented in vitro, some work concerning this phenomenon has been completed in vivo. In a study conducted in rats, it was determined that induction of the hsp's, by temperature elevation, prior to exposure to bright light resulted in a marked decrease in photoreceptor degeneration when compared to control rats <sup>67</sup>. A more recent study conducted in goldfish yielded similar results, only this time the retinas were protected from argon laser light damage subsequent to induction of the hsp's <sup>68</sup>. In this study, induction of the heat shock proteins was accomplished by placing the goldfish in an aquarium containing water heated to a sublethal temperature (35°C for 15 min). This caused induction of hsp's within the retina (as well as other regions of the body) as a result of whole body hyperthermia. When the treated retinas were exposed to 1 sec exposures from an argon laser beam, a significant degree of protection of photoreceptors resulted from the elevated levels of hsp's within the retina.

The relationship between hyperthermia-induced heat shock proteins and thermotolerance is still controversial. A study conducted in Morris hepatoma cells suggests that the expression of hsp's and thermotolerance in this cell line are most likely regulated by some interrelated mechanisms, but that hsp synthesis is neither a sufficient or necessary condition for thermotolerance development <sup>69</sup>. A more recent study conducted in 1988 has suggested that hsp70 is not required for acquisition of thermal resistance in mouse

plasmacytoma cells <sup>70</sup>. It has also been recently documented that thermotolerance can be uncoupled from induction of heat shock proteins <sup>71</sup>. In this study a mutated cell line of *Saccharomyces cerevisiae* was used. These cells lacked the ability to activate the heat shock transcription factor. Although induction of the hsps was impossible in this cell line, the acquisition of thermotolerance was still evident when cells were pretreated and then exposed to lethal elevations in temperature. The discrepancy between these studies has not been resolved and the role of stress proteins in thermotolerance is still unclear. It is possible that studies implicating that hsps are not necessary for thermotolerance to occur may not be detecting subtle changes in elevated hsp synthesis. Most studies looking at elevated levels of hsps are most likely looking at cells that are experiencing an exaggerated response, and therefore detection of hsps is quite simple. However, induction of thermotolerance may not require such high levels of hsps. In studies in which hsps are not detected but thermotolerance is achieved, it is quite possible that increased levels of hsps have been achieved, but at such low levels that detection has become difficult to nearly impossible.

Although acquired thermotolerance may be linked to the induction of heat shock proteins, the molecular mechanism(s) by which resistance is acquired is unknown. It has been suggested that the increase in  $Ca^{++}$  uptake experienced by cells placed under thermal stress may play a role in the induction of thermotolerance <sup>72</sup>. In his study it is suggested that since  $Ca^{++}$  levels return back to normal once the response has subsided, the  $Ca^{++}$  may serve as a messenger for induction of thermotolerance. Another suggested mechanism involves the rate of recovery of rRNA synthesis <sup>73</sup>. The data from this study argue that enhanced recovery of protein and rRNA synthesis can be one of the factors involved in the induction of thermotolerance.

In addition to Polla and Anderson's study (1987), several other studies were conducted in which laser tissue interaction was closely monitored and documented. These studies do not mention heat shock protein induction, but give insight into laser-tissue interaction.

It has been suggested that disorders which respond to laser therapy may be associated with the increased proliferation of cells



surrounding the injuries <sup>74</sup>. Argon laser radiation (488 nm) has been shown to increase the proliferation of cancerous cell lines (HT29, MCF17, M14, and JR1) in vitro. It was also discovered that irradiation from HeNe lasers increased the growth rate of human fibroblasts <sup>75</sup>. Similar results were observed when cell lines were exposed to Nd:YAG (1066 nm) and ruby (694.3 nm) laser radiation <sup>74</sup>.

Absorption of laser radiation in tissue has been investigated <sup>76</sup>. It has been proposed that the penetration of laser radiation into tissue is limited to a maximum of about 3 mm, with the exception of the eye. Maximum penetration into biological tissue occurs at wavelengths in the range of approximately 800-1000 nm. As the tissue absorbs laser light energy, localized heating occurs. This leads to conduction and convection of heat into surrounding tissue. Heat convection, mainly by blood flow, occurs only in well vascularized tissue and after relatively long exposure times. Many medical laser applications and retinal hazards are based on coagulation of tissue after conversion of the absorbed energy into heat. This is based on pulsed laser exposures of more than 1 ms <sup>77</sup>. A medical example of useful laser-tissue interaction involves the application of lasers in the removal of red port wine birthmarks <sup>78</sup>. In these birthmarks, hundreds of extra blood vessels that lie just beneath the skin surface can be thermally destroyed by an argon laser. This process involves photocoagulation and occlusion of the vessels which later degenerate and disappear. This application can also be used to repair bleeding vessels in retinas, seal ruptures in lungs, etc.

Although lasers serve many useful purposes, safe use and safety standards are important concerns for many users. Several studies have documented damaging effects of laser exposure to ocular tissues. Belkin and Schwartz studied the effects of extensive HeNe laser radiation on corneal epithelium and determined that an exposure of 30 sec. or more resulted in severe epithelial damage <sup>79</sup>.

Although coherent light emitted by lasers poses a threat to the retina for those at risk to exposure, of particular concern is the effect of damaging incoherent UV radiation on the eye. Short wavelength UV radiation primarily affects nucleic acid constituents of the cell, with the end result usually being cellular death. It is known that depending upon the exposure parameters involved, UV radiation may affect the cornea, the lens and/or the retina <sup>80</sup>. UV wavelengths less than 300nm can be absorbed effectively by the cellular layers of the

cornea and the skin. This results in disruption of the turnover of the epithelial layer, resulting in a partially denuded epithelial layer. This thinning occurred as damaged surface cells were prematurely sloughed off into the tear layer. Also, cell division in the basal layer was initially depressed for several hr as a result of the UV insult. The lens of the eye absorbs UV wavelengths in the range of 365 nm. With sufficiently high irradiance levels, it is possible to cause immediate damage to the lens. This causes lens opacities (cataracts) that in the longterm can cause blindspots in the field of vision. The hazard to the retina from laser radiation or from any other collimated source is the focusing of the image to a small spot on the retina, creating a much higher power density at the back of the eye than was incident at the cornea. The damaging effects of this exposure can be seen primarily in degeneration of photoreceptor inner segments. Zuchlich accomplished his work primarily on rhesus monkeys. Other investigators working on aphakic eyes observed significant damage to the RPE as well as to the photoreceptors when retinas were exposed to near UV radiation <sup>81</sup>.

### Discussion of Results

The work presented here is the first to investigate the relationship between the damaging effects of CO<sub>2</sub> laser radiation on human cells after exposure and to determine how increased synthesis of heat shock proteins is related over time. The 10.6  $\mu$ m wavelength of the CO<sub>2</sub> laser is appropriate for studying induction of the heat shock proteins since cell surfaces, mainly the surface-water interface, have a high absorptive coefficient for this wavelength. As the thin surface film of water over and within the cell absorbs the laser energy, the temperature of the interior of the cell rises, resulting in a thermal insult.

Sodium arsenite induction of the hsps was chosen as the positive control in this study. This sulfhydryl reagent will bind to the cysteine residues of proteins in such a manner as to denature these proteins, thereby stressing the cells and inducing the stress response <sup>13</sup>. It has been suggested that abnormal proteins serve as a trigger for the induction of the stress response <sup>17, 82</sup>.

Irradiation of the human fibroblasts in this study resulted in the increased synthesis of hsp70 as a result of this insult. Fluorographic studies indicated that hsp70 levels increased within 2 hr after induction of the response. The response peaked at 4 hr, and by

30 hr was back down to baseline levels. Only increased levels of hsp70 were observed, indicating that hsp70 may be regulated independently of the other hsps. This also suggests that one possible effect of the laser with respect to the induction of hsps may be a wavelength phenomenon rather than a thermal effect.

Arsenite induction of the heat shock proteins was quite different from that of laser induction. One of the differences was that not only was hsp70 induced, but so was hsp32. Also, the response at 24 hr was nearing baseline levels, but at 30 hr methionine incorporation again peaked. Arsenite is therefore a more efficient inducer of the heat shock proteins. It is quite possible that since arsenite is an extremely stable compound, it remains within the cell, exerting its effects on sulfhydryl groups of proteins and continuing to be turned over as proteins are discarded by the cell. Laser radiation, on the other hand, is a pulsed insult. The cells are intensely heated for a brief moment and then heat is dissipated. It is believed that hsp70 may serve as an intracellular "thermometer" <sup>83</sup>. This review by Craig and Gross suggests that hsp70 regulates the expression of all heat shock proteins in response to a thermal insult. Since hsp70 is the only stress protein induced by CO<sub>2</sub> laser radiation, it may be possible that hsp70 is actually inhibiting the synthesis of other heat shock proteins in response to brief thermal insults. The exact mechanism of this inhibition needs to be studied in greater depth. Arsenite induction of hsp70 probably occurs by a different mechanism than by laser pulse induction, and therefore hsp70 does not appear to be an inhibitor of the synthesis of other hsps when induced by arsenite.

This study also investigated the intracellular localization of two major subclasses of hsp70, hsp72/73. Immunolocalization of hsp72/73 was determined by utilizing a monoclonal antibody against both hsp72 and 73. The detection system used was the streptavidin/gold with silver enhancement technique as described in the materials and methods. Whereas radiolabeling and fluorography evaluates newly synthesized proteins, immunolocalization identifies not only newly synthesized proteins and information about cellular location but also the accumulation of proteins at different time intervals.

Within the first four hr after induction by the CO<sub>2</sub> laser, the majority of the hsp72/73 is localized to the nucleus. This is also true of arsenite induced cells. This indicates that the DNA within the cell may be vulnerable to damage during transcription of RNAs, and the

presence of hsp70 within the nucleus may be necessary to assist the cell in the shutdown of ribosomal assembly during this critical point in the cell cycle. Hsp70 may also be there in order to turnover any denatured protein that may exist as a result of the stress. It was discovered by Lewis and Pelham that the 70kD stress protein, when induced by heat shock, will bind tightly first to nuclear components and then to nucleoli <sup>84</sup>. This study further supports the importance of hsp70 in the restoration of nuclear function once it has been diminished by noxious insults.

A classic study carried out by Welch and Suhan utilized an indirect immunofluorescent system for detection of hsp70 in rat embryo fibroblasts <sup>43</sup>. This study determined that approximately four hr after recovery from heat shock, the majority of hsp70 resides within the nucleus, with large amounts present within nucleoli. After eight hr of recovery, the nucleoli slowly regain their normal morphology and the 70kD stress protein exits this organelle. The 70kD stress protein then begins to accumulate within the cytoplasm, mainly in the perinuclear and plasma membrane regions. By 24 hr of recovery, much of the heat shock protein is no longer detectable and nuclear morphology has returned to normal. Several other researchers utilizing immunofluorescence have also detected hsp70 in HeLa cells <sup>57, 85</sup>, Drosophila cultured cells <sup>86</sup>, and rat cardiac myocytes <sup>87</sup>. One study utilizing immunoperoxidase staining even detected HSP70 in atherosclerotic specimens of human arteries <sup>88</sup>.

At 8 hr post induction in our study, the laser-treated cells continue to exhibit heavy nuclear labeling, whereas the labeling in the arsenite-treated cells had shifted to the cytoplasm. This indicated that the nuclear damage caused by the CO<sub>2</sub> laser was more extensive than the damage produced in arsenite-treated cells. By 12 hr, the labeling in the laser-treated cells had finally moved to the cytoplasm. Arsenite-treated cells were similar in appearance at this time point. At 24 and 32 hr post induction, both sets of cells indicated cytoplasmic localization of hsp72/73. The difference occurs at 48 hr. Although newly synthesized hsp72/73 had decreased to baseline levels by 30-32 hr, heavy localization of hsp72/73 continued to persist in both the laser and arsenite-treated cells. This indicates to us that although the synthesis of hsp72/73 is back to normal at 30 hr post induction, hsp72/73 continues to exert its effects on the cell well after the synthesis of hsp72/73 had diminished. This suggests that cellular damage persists well after the diminished synthesis of hsps such that hsp72/73 must remain within the cell to continue to reestablish

normal cellular functions. It was apparent, however, that the labeling of hsp72/73 in the laser-treated cells at 48 hr had somewhat subsided, indicating that the stress response had begun to diminish somewhat. In the arsenite-treated cells, however, heavy labeling persisted at 48 hr, and again, this was probably due to the stability of arsenite and its continued effects upon cysteine residues of proteins within the cell.

To gain a better understanding of the actual effects of the CO<sub>2</sub> laser upon cells in culture, this study investigated the possible effects of pulses of hot media on the stress response. In consultation with physicists at LAIR, physical calculations estimated that the temperature reached by the cells during induction by the laser was approximately 67°C. Therefore, 1-2 second exposures of 70° C media were used as a reference point for possible induction of the stress response. Pulses of 70° C media for 1-2 sec elicited a very pronounced induction of heat shock proteins when evaluated immunocytochemically. This response, however, differed in one significant aspect. The labeling that occurred in cells pulsed by hot media 8 hr post induction was primarily cytoplasmic. The laser-treated cells exhibited nuclear labeling at 8 hr post induction. This suggested: 1.) The effects of the laser are primarily thermal since the experiment using pulses of hot media induced a similar response, 2.) the particular wavelength effects of the laser upon induction of hsps cannot be ruled out since the localization of hsp72/73 differed between the two experiments, and 3.) that nuclear damage is more pronounced when cells are exposed to brief pulses of CO<sub>2</sub> lasers than when exposed to brief pulses of hot media. The reason for increased nuclear damage may be due to the high peak powers obtained in the center of the exposed cell area. Lower temperatures of hot media elicited little or no localization of hsp72/73 within the cells. It is also necessary to note here that the actual temperature of the media must have been a few degrees lower measured in the culture tube because the media cooled during pipetting into the 24-well multiplate. Also, this experiment does not actually simulate the effects of the laser. The duration time for exposure of the cells to high temperature with the use of hot media is approximately 1-2 sec. Laser exposure was for .8 sec. This study provides some insight as to the possible effects of infrared wavelengths of light (10.6  $\mu$ m) upon cells in culture with respect to induction of heat shock proteins.

Results suggest the following conclusions :

- 1) The increased synthesis of hsp is induced by .8 sec pulses from a carbon dioxide laser.
- 2) The major heat shock protein induced is hsp70.
- 3) Hsp70 is localized to the nucleus during the early stages of induction of hsp70 and to the cytoplasm during later periods of recovery.
- 4) Induction of the synthesis of hsp70 is due primarily to the thermal effects of the carbon dioxide laser.
- 5.) The shortest time and highest temperature yet reported to induce the heat shock proteins was observed in this study.
- 6) Data provides additional information on laser tissue interaction.

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